African Journal of Biotechnology Vol. 10(47), pp. 9606-9612, 24 August, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.850
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Acibenzolar-S-methyl induces lettuce resistance against *Xanthomonas campestris* pv. *vitians*

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Accepted 4 July, 2011

Acibenzolar-S-methyl (Benzo [1,2,3] thiadiazole-7-carbothioic acid-S-methyl ester, ASM; Bion 50 WG) was found to be more protective for lettuce against bacterial speck disease caused by *Xanthomonas campestris* pv. *vitians.* The experiments were conducted under controlled climatic conditions in the greenhouse, and induction of defense mechanism using ASM treatment against challenged inoculation with *X. campestris* pv. *vitians* was studied. It was effective in reducing the severity of disease and bacterial growth when compared with control and copper hydroxide. Pathogen growth was also followed with specific PCR before the typical symptom development on leaf tissues. Equal plant tissues collected from infected pnjm, lants were used in order to compare different treatments and pathogen invasion. The induction interval (48) before inoculation period was convenient for the control of pathogen by ASM treatment. The findings show that ASM treatment in inoculated plants has long lasting effect to induce defense-related enzymes, contributing to the enhancement of plant resistance. The effect was comparable with copper treatment. As a marker of resistance, PR protein activity chitinase showed remarkable increase, depending on decreasing bacterial growth *in planta*.

Key words: Acibenzolar-S-methyl, induced resistance, *Xanthomonas campestris pv.vitians.*

INTRODUCTION

Bacterial leaf spot (BLS) leads to economic losses in lettuce growing fields in southern Turkey. The disease has previously been reported in Turkey (Sahin, 2000).

There are two discrete symptoms associated with BLS. One of them causes watersoaked, brown lesions that later turn black, and they are about 1 to 2 mm in diameter. These lesions have V-shape, are translucent and they collapse (Toussaint, 1999; Sahin and Miller, 1998). Lesions may expand along the veins of the plant (Sahin and Miller, 1997; Toussaint 1999; Wallis and Joubert, 1972). Another type of symptom consists of small black spots scattered along the leaf surface (Sahin and Miller, 1997). The differences in symptom development are associated with the type of pathogens and this case has been evaluated with sequence comparisons (Myung et al., 2010). Bacterium can be dispersed by infected seed (Ohata et al., 1982; Pernezny et al., 2001; Umesh et al., 1996). A few infected seeds can lead to many diseased seedlings and may mean that the pathogen can cause significant field epidemics when even low rates of seed transmission occur. *Xanthomonas c.* pv. *vitians* can survive in soil associated with plant debris (Barak et al., 2001).

Control of this pathogen is difficult, and since the copper compounds are frequently used, they have hazardous effect on human health and cause environmental polution. Therefore, one of the alternative control methods is to induce the plant resistance to pathogen.

Induction of plant's defense genes by prior application of inducing agents is called induced resistance (Hammerschmidt and Kuc, 1995). Systemic acquired resistance (SAR) is characterized by a reduction in the number of lesions and disease severity caused by challenged inoculation with pathogen (Hammerschmidt, 1999). Recently, acibenzolar-S-methyl (Benzo [1,2,3] thiadiazole-7-carbothioic acid-S-methyl ester (ASM) is developed as a potent SAR activator which does not

Abbreviation: ASM, Acibenzolar-S-methyl; **SAR,** systemic acquired resistance.

Figure 1. Symptom of *X. campestris* pv. *vitians* on lettuce.

have antimicrobial properties. Instead, it increases crop resistance to disease by activating the SAR signal transduction pathway on several plant species besides the natural products such as salicylic acid (SA) (Kessmann et al., 1994; Anfako, 200; Reuveni et al., 2001). The development of SAR is often associated with the various cellular defence responses such as synthesis of pathogenesis-related (PR) proteins, phytoalexins, accumulation of active oxygen species (AOS), rapid alterations in cell wall and enhanced activity of various defence-related enzymes (Ryals et al., 1996). Development of alternative control measures is necessary to control this disease. Recently, chemical elicitors are increasingly used for plant diseases control. The mechanism of action of chemical elicitors inhibits pathogens by stimulating systemic acquired resistance reactions in plants (Lucas, 1999). One compound that meets these criteria is acibenzlar-s-methyl (BTH, 1,2,3-benzothiadiazole-7-thiocarboxylic acid s-methyl ester) which is marketed as Bion® or Actigard™ by Syngenta Cooperation and is shown to elicit SAR against numerous fungal, bacterial and viral pathogens (Oostendorp et al., 2001). This compound has been studied as the principle component of disease management strategies in a number of field experiments (Standik and Buchenauer, 1999; Gorlach et al., 1996) and is recently being marketed for controlling bacterial spot and scab diseases of tomato, downy mildew of spinach and blue mold of tobacco in USA (Moffat, 2001).

Until now, no information is available regarding physiological changes during induced resistance of ASM against the *X. c.* pv*. vitians* on lettuce. In this study, the synthetic inducer ASM was studied for its potential effect against pathogen.

MATERIALS AND METHODS

Plant material

Greenhouse-grown lettuce plants (Arizona RZ) were used for all experiments. These are susceptible to *X. campestris* pv. *vitians*. Plants were grown in 10 cm pots containing one plant in a soil mix of sand, perlite and peat compost in the greenhouse at 25 ± 5°C with 68 to 80% RH. The soil mix also contained a slow-release fertilizer (14-12-14 N-P-K). Plants were watered daily. Light was supplemented by a single 1000-watt sodium vapour lamp during a 16 h photoperiod. The plants were used 4 weeks after planting (young shoots were 10 to 12 cm long with 6-8 leaves per shoot). These conditions were maintained during the experiment.

Bacterial strain and inoculation

Bacterial strain of *X. campestris* pv.*vitians* (6/10) was isolated from infected seedlings and stock culture was preserved on the NYA medium in glass flasks at 4°C in a refrigerator. The bacteria were transferred every 3 month to new flasks. Inoculum suspension was prepared from early log-phase cells which were obtained by growing the bacterial strain in nutrient broth in 25 ml sterile tubes and incubated at 27°C on an orbital shaker at 200 rpm for 24 h.

Bacteria were subsequently pelleted by centrifugation (twice, each at 3500 × *g* for 5 min) and washed in sterile distilled water (SDW). Their concentration was adjusted to 10^8 cfu/ml by dilution to give OD_{660} of 0.1. The leaves of the seedlings were inoculated by spraying a suspension of 10^8cfu/ml onto plant leaves.

Inoculum preparation and plant inoculation

X. c. pv. *vitians* strain isolated was used in these tests. Colonies on peptone sucrose agar were yellow and raised with smooth margins. Pathogenicity was evaluated on 3-week-old lettuce plants. Bacteria were grown on TSA for 48 h at 28°C. A bacterial suspension in sterile distilled water (100 ml at 1 × 10^5 CFU/ml) was sprayed onto

three plants for each isolate. Plants were maintained in a growth chamber at 28°C and 90% relative humidity. Isolates caused identical symptoms 3 days after inoculation as those originally observed in the fields (Figure 1). The pathogenicity of bacteria re-isolated 10 days after inoculation from lesions surface sterilized in 70% ethyl alcohol was confirmed by inoculation as described earlier. No symptoms were observed on two control plants treated with sterile distilled water. The pathogen was grown for 3 days at 28°C on nutrient agar (NA) plates. Bacterial suspensions were adjusted turbidimetrically to ca. 1×10^8 colony forming units (CFU) /ml using a spectrophotometer. Suspensions were diluted to 1×10^7 CFU/ml. Twenty plants of each treatment, between 4 to 6 weeks old, were sprayed with the bacterial suspension until they were run-off on both the adaxial and abaxial surfaces using a hand-held pump sprayer. Controls consisted of two lettuce plants sprayed with sterile tap water and placed at each temperature. All plants were immediately encased in clear plastic bags. After 3 days, bags were removed and plants were arranged in a completely random design in the growth chamber. Each treatment was arranged in three replications.

Application of ASM and copper compound

ASM (Bion®, Syngenta, Frankfurt, as 50% active ingredients in WP formulation) was dissolved in distilled water to obtain concentration of 0.2 mg/ml and then sprayed on whole seedlings (ca. 200 µl per seedling). After the treatment, seedlings were maintained in a greenhouse as described earlier. Control plants were sprayed with water at the same intervals. The cupper hydroxide (consisting of 1.5% active cupper) treatment was also sprayed as positive control. The most efficient induction interval time conferred by ASM was assigned in equal numbers to three treatment groups.

Effect of ASM on bacterial growth *in planta*

Bacterial colony forming units (cfu) were recovered from inoculated tissues, treated with either ASM or water 3 days before inoculation. This was done by removing inoculated shoot tips (1 g plant material). These units were collected and homogenized in 0.06% NaCl solution (1:1) diluted serially from 10^{-1} to 10^{-6} and was performed on the modified Miller-Schroth medium. Aliquots of alternate dilutions were plated onto NYA agar plates. Plates were incubated at 27°C for 48 h and emerging colonies were counted on all dilution plates showing bacterial growth. Bacterial numbers *in planta* were calculated for each of the dilution plates and a mean value was obtained from the replicates. Each dilution from each leaf disc was duplicated. Results presented are means for two separate experiments in which three leaf discs were homogenized from each treatment.

Confirmation of the pathogen by specific PCR

To confirm the identity of Xanthomonad-like colonies recovered from lettuce plants, *X. campestris* pv. *vitians*-specific primer pair (B162) was designed as B162 left 5' CGG CCG AAT AAT CAA GAG CA 3' and B162 right, 5' TTG TCC GTT ACG ATT GGT GC 3' was used to confirm PCR (Barak et al., 2001). The template for the PCR was a boiled cell extract prepared from a bacterial cell suspension. This was made by adding a loopful of bacteria from an individual colony into 1 ml sterile water in a 1·5 ml Eppendorf tube. The tubes were boiled for 10 min, and 2 µl of this cell extract was used in the PCR. The PCR was carried out in a Thermal Cycler 480 (Perkin-Elmer, CA, USA). The amplification profile was 35 cycles of 94°C for 1 min, 55°C for 2 min and 72°C for 3 min. Amplified DNA fragments that directed the amplification of an approximately 700-bp DNA fragment from total genomic DNA of *X. campestris* pv. vitians were analysed by gel electrophoresis in 1·5% agarose in 0·5 × Tris-borate EDTA buffer. Gels were stained with ethidium bromide and DNA was visualized with a gel-imaging system.

Diseases severity per each treatment, experimental design and statistical analyses

Twelve days after inoculation, disease severity was evaluated by rating the most severely damaged area of all the treated plants on a scale of 0 to 5 (plants with no visible symptoms = 0; a few individual lesions = 1; many individual lesions = 2; small patches of coalesced lesions = 3; medium sized patches of coalesced lesions = 4; and large patches of coalesced lesions = 5) and calculated with Tousend-hoberger equation (Bull and Koike, 2005). The experiments were arranged in a completely randomized design with there replications. Each replication consisted of 20 plants.
The experiment was repeated twice. Standard analyses of variance (ANOVA) were carried out using the SPSS Statistical Package, Version 11. ANOVA was performed to analyze the data, and the significance of differences among treatments was determined according to Duncan and Tukey's multiple range test ($P < 0.05$).

Determination of chitinases activity

Leaves treated with ASM, copper hydroxide or water were inoculated with the *X. campestris* pv. *vitians*, 2 days after the induction. For controls, plants were sprayed with water but not inoculated. Both inoculated and uninoculated leaves were removed at the indicated periods. Chitinases activity was determined by the method described by Baysal and Zeller (2004). High polymeric carboxymethyl-substituted chitin, labelled covalently with Remazol Brilliant Violet 5R (CM-Chitin-RBV, Loewe, Biochemica, Germany) was used as substrate for chitinase activity. Potassium acetate buffer (0.2 ml, 0.1 M and pH 5.0) and 0.1 ml of suitably diluted crude extract were added to a micro-centrifuge tube and allowed to equilibrate to 37°C. The reaction was initiated by adding 0.1 ml aqueous CM-Chitin-RBV (2 mg ml^{-1}). The reaction was terminated by adding 0.1 ml 2 N HCl, which precipitated the non-degraded substrate. Tubes were cooled on ice for 10 min and then centrifuged for 5 min at 9000 *g*. Absorbance of the supernatant at 550 nm was recorded and the results were calculated as a change in optical density at 550 nm mg $protein^{-1}$ min^{-1}. Blanks were prepared similarly with Na-acetate buffer instead of the homogenate. Enzyme activity was expressed in µmol of the reaction product min^{-1} mg^{-1} protein. Each sample of extract was measured twice in each replicate and at least two replications were performed per each reading.

RESULTS

The effect of the ASM treatment on disease

Among the different induction interval tested, 2 days before inoculation increased plant vigour and consistently reduced the disease severity under greenhouse conditions in comparison to other induction intervals (data not shown). After application of ASM, a remarkable reduction in the disease index of *Xcv* occurred (Table 1). The resistance induced by the ASM-treatment was evident at the 7th day and lasted for the entire experimental period (until the 11th day). Untreated plants

Table 1. Mean disease severity in lettuce treated with ASM and cupper hydroxide.

Treatment	Disease severity[1]
ASM	21.8[A]
Cupper hydroxide	16.8[A]
Control	61.5[B]

Means followed by the same letters are not significantly different ($P < 0.01$).

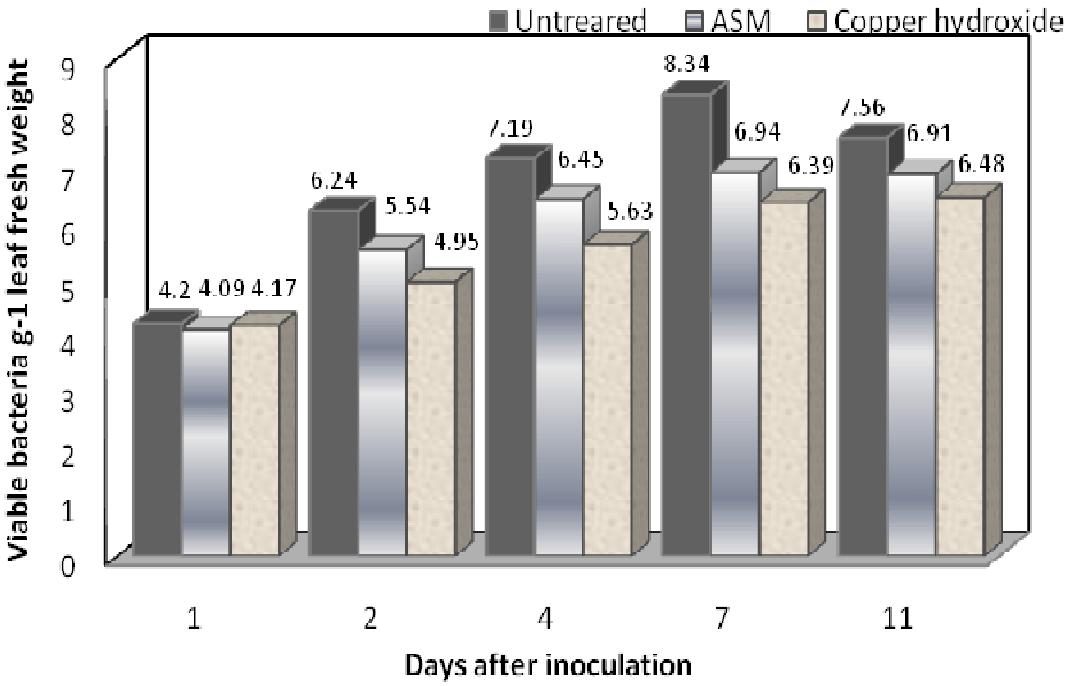

Figure 2. Effect of ASM treatment on the bacterial growth of *X. campestris* pv. *vitians*. The leaves treated with ASM or water (control). The plants were inoculated 2 days after ASM treatment. Log data are the mean of independent two experiments.

showed rapid disease development during this period compared to control. Disease severity was reduced by 75% in ASM treated seedlings at day 7 and the less symptom development was maintained until the 11th day. Disease severity of control seedlings was 61.5%, whereas those of ASM treated seedlings were only 21.8% at day 11 and 72% was in copper hydroxide treated plants.

Growth of bacteria after ASM, copper hydroxide spray and its following by specific PCR

ASM treatment led to a remarkable decrease on the growth of *Xcv* in plant tissue (Figure 2). The time between initial treatment with ASM and subsequent inoculation with *Xcv* significantly affected efficacy of the induced resistance. This inhibitory effect was first observed in day 4 and monitored until day 7. The greatest decrease on bacterial growth occurred at the 7th day and lasted for the entire experimental period (11th day). Untreated plants showed rapid bacterial growth during the whole period compared to ASM and copper hydroxide treated plants. Bacterial population decreased by 75% in ASM treated seedlings at day 7 and this ratio was 82% in copper hydroxide treated plants. This suppression on bacterial growth was maintained until the 11 day (Figure 2).

The PCR confirmation results showed partly correlation with the growth of the pathogen *in planta*. On treated lettuce showing less symptom than that of control, plants obtained from excised plant tissues clearly exhibit recession of bacterial growth on plants, depending on ASM treatment and copper compounds. ASM treatment has displayed its efficiency in 2 days inoculation, whereas copper plants showed this effect in 1 day inoculation

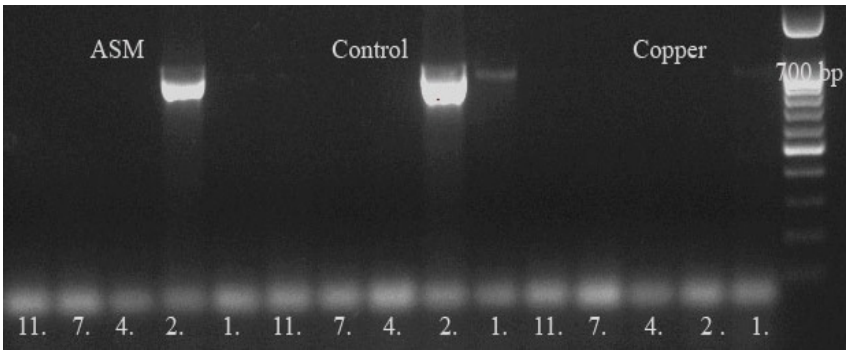

Figure 3. The confirmation of *X. campestris* pv. *vitians* by PCR in equal plant tissue. Effect of two treatment on the bacterial growth of *X. campestris* pv. *vitians*, compare with control. Leaves were treated with ASM, cupper oxide or water (control). The plants were inoculated 2 days after ASM treatment, but copper was applied at the same day after inoculation.

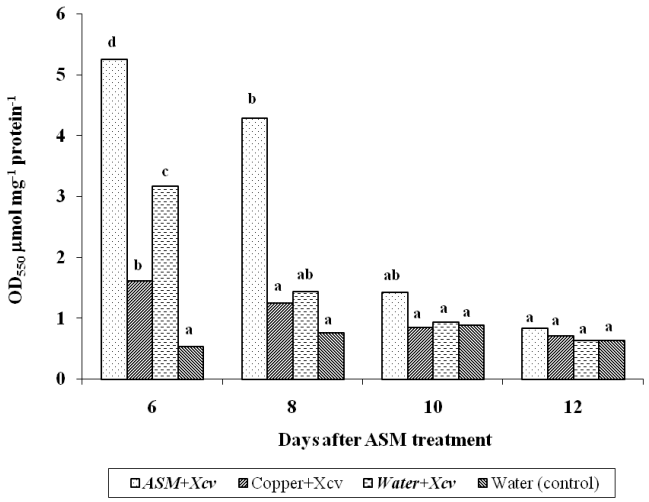

Figure 4. The effect of the ASM treatment on the activity of chitinases on lettuce leaves. The results are expressed as the mean of two separate experiments (in each experiment two different extractions were pooled at every time point). The values with the same letters represent values that are not significantly different according to Duncan's multiple range test ($P < 0.05$).

(Figure 3). But there was no- PCR priming on both treated and control plants 4 days after inoculation. This is because the typical symptoms on excised plants were low while diseased leaves were being collected during the experiments.

Chitinase activity

Treatment of seedling with ASM followed by inoculation with Xcv resulted in the marked increase in chitinases activity (Figure 4). The activity of chitinases in these plants was significantly higher than those observed in two treatments, 6 and 8 days after induction. As shown in Figure 4, the activities observed by 6 and 8 days were significantly different from water-treated control plants (inoculated). The levels of chitinases activity remained low in both inoculated and uninoculated water-treated seedlings during the time course (Figure 4).

DISCUSSION

Plants have endogenous defence mechanisms that can be induced in response to attack by insects and pathogens. One of the potential methods of reducing the severity of disease caused by the pathogen is induction of resistance, achieved through the use of chemicals or

biotic inducer agents (Sticher et al., 1997). Certain chemicals such as ASM, SA, INA and BABA can induce resistance. Subsequent experiments revealed the ASM to be a potent SAR inducers providing protection against a wide spectrum of plant pathogens (Lawton et al., 1996).

For the development of resistance plants, there needs to be an interval period before pathogen inoculation. This interval was reported between 2 and 7 days in most cases and the pre-inoculation of plants with avirulent pathogens or abiotic elicitors was assessed for induction of resistance against several plant diseases (Baysal et al., 2003). This effect was seen 1 to 3 days after inducer application (Schweizer et al., 1999). In accordance with our study, synthetic chemical 2,6-dichloroisonicotinic acid treated plants disease resistance against fungal diseases occured five days after treatment (Mètraux et al., 1990); and 1-2 days after treatment with oxalate and sodium phosphates treatment gave rise to a resistance (Doubrava et al., 1988; Gottstein and Kuc, 1989; Mucharromah and Kuc, 1991). In our study, the highest disease resistance was observed on treated plants and the occurrence of resistance emerged 3 days after ASM treatment compared to untreated ones.

In previous studies treatments with synthetic chemicals, for instance 2,6-dichloroisonicotinic acid derivates were tested against fungal diseases; protection was achieved against *Collectotrichum lagenarium* two days after induction, while protection against *Cercospora beticola* was achieved five days after induction (Metraux et al., 1990). In this study, although different induction times in ASM treated plants showed a low disease severity, the best induction time against *Xcv* was found to be three days before inoculation. During the here reported greenhouse experiments in ASM treated plants, the slower symptom development was correlated with a considerably lower bacterial populations compared with the untreated ones. In addition to suppresion of disease development, an evaluation on whether ASM treatment has any effect on the control of bacterial growth in plant leaves was done. Findings also revealed a remarkably reduction in the degree of infection correlated with a considerably lower bacterial growth in treated plant leaves. These data indicate, that the resistance inducers did not only suppress symptoms but also directly or indirectly inhibited the pathogen`s multiplication in planta. These results were confirmed with PCR analysis from the plant tissue directly collected from equal weight of plant tissue. On PCR, getting a decrease in specific bands obtained from the plants showing typical symptom can be associated with excising the diseased plant parts from day to day and its effect on bacterial recession was estimated per fresh plant tissue. This could be the reason for reducing the bacterial population to a level below the detection level. The other main concern has been reported as primer reactivity (that is sensitivity, specificity and accessibility), resulting from “false negatives” due to low sensitivity. The sensibility level of PCR showed change from 10^2- 10 CFU/ml (Mills et al. 1997). This can be valid for the primers used in BLS disease detection.

On the other hand, the reduction of bacterial multiplication in ASM treated plants was also accompanied by physiological changes in the plant’s tissue after induction. However, 11 days after inoculation the bacterial population in ASM treated plants did no longer show a considerable difference to untreated plants. From this observation it may be concluded that the used resistance inducers have an effect only up to 7 days pi. Also in other host/parasite-interactions a correlation between the reduction of bacterial populations and development of resistance in ASM treated plants has been reported (Lawton et al., 1996; Siegrist et al., 1997). These results are in accordance with other studies on resistance mechanism where the development of symptoms was delayed and the multiplication of bacteria was on a low, but constant rate (Lozano and Sequeira, 1970; Goodman et al., 1986). A low nutrient concentration in the intercellular space can be a limiting factor for the growth of bacteria. But also apoplastic compounds or antimicrobial metabolic components of plants with bacteriostatic properties may be involved in the control of bacterial growth in ASM treated plants (Siegrist et al. 1997). In plants, development of induced resistance is associated with the co-ordinate expression of a complex set of so-called ‘SAR genes’ which include genes for some of the pathogenesis-related (PR) proteins (Conrath et al., 2001). The enzymatic activities of several PR proteins have been identified as chitinases (PR-3), which possess direct antimicrobial activity by degrading microbial cell wall components (Stintzi et al., 1993). It is known that some plant chitinases have lysozyme activity also and therefore, can hydrolyse bacterial cell walls (Boller et al., 1983; Heitz et al., 1994). These findings have also been confirmed in previous studies carried out on tomato (Baysal et al., 2003).

In ASM treated seedlings, significantly lower bacterial population was observed up to 96 h post inoculation compared to untreated plants. Bacterial growth of *P. syringae* pv. *tomato* was slightly inhibited by ASM *in vitro* conditions as reported by Scarponi et al. (2001). The findings suggest that the protection of tomato seedling from pathogen must have been due to the activation of the plant defense mechanisms. It may be hypothesized that a low nutrient concentration or/and accumulation of antimicrobial compounds in the intercellular spaces of treated tomato leaves, where bacteria grow, or where cell wall alterations occur as a physiological barrier in xylem tissues may be limiting factor for bacterial growth as a consequence of the treatment; and this may be responsible for the reduction in disease severity as in the case in other studies. Reduced bacterial growth was also reported in Arabidopsis, bean and tomato plants treated with ASM and challenged with *P.s*. pv. *tomato* and *X. axonopopodis* pv. *phaseoli* (Goodman et al., 1986; Lawton et al., 1996; Siegrist et al., 1997; Scarponi et al.,

2001).

In conclusion, ASM is proved to be an efficient activator of several plant defense mechanisms. Regarding the fact that the ASM dependent resistance does not appear due to an antimicrobial effect of the compound, ASM may represent a useful tool for inducing resistance in lettuce as observed in other plant species. Although, ASM treatment does not seem to be a certain control measurement in greenhouses against BLS, it can be concluded that ASM is responsible for a stimulation of resistance in the host plant and can accelerate a defence response to stop bacterial migration in the plant tissue. Hence, with ASM treatment, a bit long period is to be ensured for the intensive agricultural areas until the end of harvest season; instead of eradicating plants planted in greenhouses. The results clearly suggest that ASM is a beneficial tool in controlling BLS disease in lettuce growing areas. ASM can be assesed using low doses combination of copper compounds in management of the BLS disease.

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